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14. ABSTRACT

This study combines the TGFbeta neutralizing antibody, Fresolimumab, with Radiation Therapy (RT) to treat metastatic breast cancer. Fresolimumab is administered intravenously (i.v.) at either 1mg/kg or 10mg/kg on day 1 of weeks 0, 3, 6, 9 & 12 and RT administered at 7.5 Gy/fraction in 3 fractions during weeks 1 (to lesion 1) and 7 (to lesion 2). The primary objectives are 1) to assess safety, feasibility and tumor regression 2) to monitor immune responses in these patients and 3) to correlate immune responses with abscopal effects assessed by imaging. Blood samples are obtained from patients before, during and after treatment and immune monitoring is performed at UCLA (Figure 1). In addition, Fresolimumab is examined at UCLA for its potential effects on breast cancer stem cells.

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1 Introduction

This study combines the TGFbeta neutralizing antibody, Fresolimumab, with Radiation Therapy (RT) to treat metastatic breast cancer. Fresolimumab is administered intravenously (i.v.) at either 1mg/kg or 10mg/kg on day 1 of weeks 0, 3, 6, 9 & 12 and RT administered at 7.5 Gy/fraction in 3 fractions during weeks 1 (to lesion 1) and 7 (to lesion 2). The primary objectives are 1) to assess safety, feasibility and tumor regression 2) to monitor immune responses in these patients and 3) to correlate immune responses with abscopal effects assessed by imaging. Blood samples are obtained from patients before, during and after treatment and immune monitoring is performed at UCLA (Figure 1). In addition, Fresolimumab is examined at UCLA for its potential effects on breast cancer stem cells.

2 Body

2.1 Immune-monitoring

ACTIVE PATIENTS		IRB #11002197; PI: Percy Lee, M.D.				
Subject ID	**Initials**	**Sex**	**Race**	**Eth**	**MD**	**Date of Consent**
11002197-03	A-T	F	A	NH	PL	6/21/12
11002197-04	YOP	F	A	NH	PL	7/26/12
11002197-05	R-S	F	A	NH	PL	8/8/12
11002197-06	CAW	F	B	NH	PL	9/24/12
11002197-07	JBG	F	W	NH	PL	9/27/12
OFF STUDY PATIENTS						
11002197-01	SJC	F	W	NH	PL	5/22/12
11002197-02	B-K	F	A	NH	PL	7/10/12
SCREEN FAILURES						
Subject ID	**Initials**	**Sex**	**Race**	**Eth**	**MD**	**Date of Consent**
Not assigned	WCF	M	W	NH	PL	5/24/12

Table 1: Patients enrolled at UCLA.

At UCLA, to date we have consented 8 subjects to date, with 5 receiving treatment and one screening failure due to lab tests and progressive disease (Table 1).

The immune monitoring has been performed as described in the original application. Standard operating procedures (SOPs) for separating peripheral blood cells from plasma and the long-term storage thereof were tested using blood from 10 patients who were not part of the trial in order to provide basline values for the immune subset monitoring and analyses. Control levels were established using “normal” PBMCs that are run every time as an internal standard. The tetramer analysis has been established for CD8+ cells HLA-A2.1+ve patients as a measure of tumor-specific immune reactivity. Multicolor flow cytometry has used an antibody panel for the immune monitoring of immune cell subsets that are shown in Fig 1. Two tubes are used – one for lymphoid and one for myeloid cells. The markers are chosen to allow discrimination between

subsets that are identified in the figure, although it should be noted that the exact identity of some of these subsets is still at the stage where their function is still based on only a few publications. We use AAD as a viability marker and CD3/CD19/CD20 exclusion to provide better coverage of myeloid cells. The longitudinal nature of the trial allows us to follow immune changes with time and derive information on how radiation therapy and Fresolimumab might influence the response to cancer.

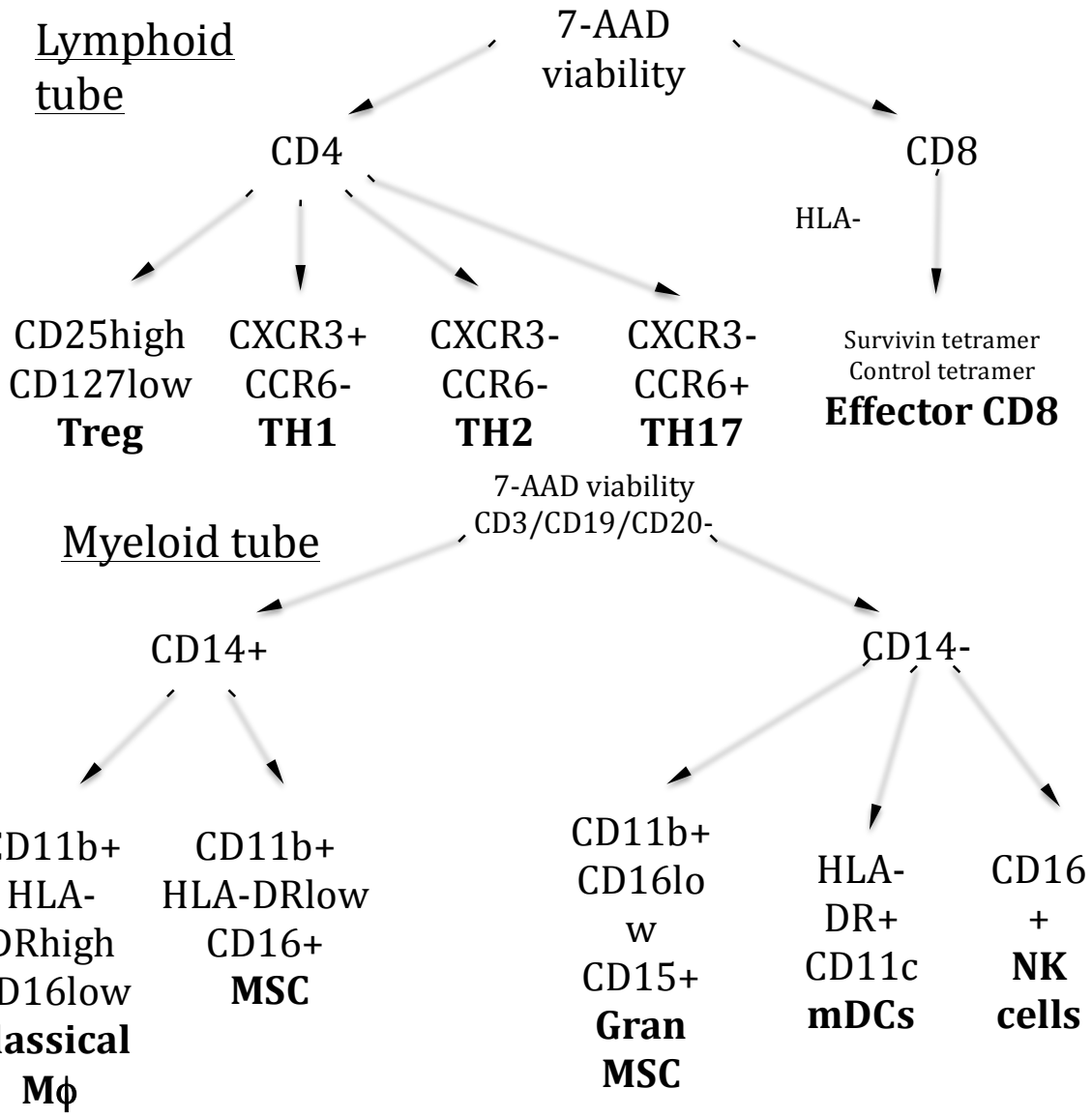


Figure 1: The schema developed for multi-color immune analysis of PBMCs.

Because NYU had started enrolling patients before UCLA, we chose to start with the samples from the patients shown in Table 2. It should be noted that we were able to recover viable PBMCs shipped to UCLA from NYU verifying that the SOPs in place were effective.

HLA-A2.1	Sample number	Date Drawn	PID	Visit	Vol blood received (ml)	Date Processed
+	11-00533B-2011-001	7/28/2011	01-BE	Wk 0 PRE	50	7/29/2011
	11-00533B-2011-002	8/11/2011	01-BE	Wk 2	52	8/12/2011
	11-00533B-2012-013	5/7/2012	01-BE	Wk 0* tx delay	40	5/8/2012
	11-00533B-2012-016	5/21/2012	01-BE	Wk 2* tx delay	50	5/22/2012
	11-00533B-2012-019	6/11/2012	01-BE	Wk5* tx delay	40	6/11/2012
+	11-00533B-2011-003	11/10/2011	02-CB	Wk 0 PRE	40	11/10/2011
	11-00533B-2011-004	11/29/2011	02-CB	Wk 2	40	11/30/2011
	11-00533B-2011-005	12/27/2011	02-CB	Wk 6 (wk 5 window)	50	12/28/2011
	11-00533B-2012-007	2/29/2012	02-CB	Wk 15	44	3/1/2012
+	11-00533B-2012-001	2/2/2012	03-HL	Wk 0 PRE	52.5	2/3/2012
	11-00533B-2012-004	2/15/2012	03-HL	Wk 2	52	2/16/2012
	11-00533B-2012-009	3/7/2012	03-HL	Wk 5	56	3/7/2012
	11-00533B-2012-014	5/14/2012	03-HL	Wk 15	54	5/15/2012
+	11-00533B-2012-002	2/7/2012	04-MM	Wk 0 PRE	45	2/8/2012
	11-00533B-2012-003	2/14/2012	04-MM	Wk 2	50	2/15/2012
	11-00533B-2012-011	3/21/2012	04-MM	Wk 5	35	3/22/2012
+	11-00533B-2012-005	2/16/2012	05-KJK	Wk 0 PRE		2/17/2012
	11-00533B-2012-008	3/1/2012	05-KJK	Wk 2	35	3/2/2012
	11-00533B-2012-012	3/22/2012	05-KJK	Wk 5	53	3/23/2012
-	11-00533B-2012-006	2/28/2012	06-MM	Wk 2	57.5	2/29/2012
	11-00533B-2012-010	3/19/2012	06-MM	Wk 5	51	3/19/2012
	11-00533B-2012-017	5/29/2012	06-MM	Wk 15	40	5/29/2012
-	11-00533B-2012-015	5/18/2012	07-KF	Wk 0	57	5/8/2012
	11-00533B-2012-018	5/31/2012	07-KF	Wk 2	45	6/1/2012

Five of the 7 patient samples that were analyzed were HLA-A2.1+ve. The tetramer results are shown in Fig 2. Note that one patient (blue lines) was treated twice.

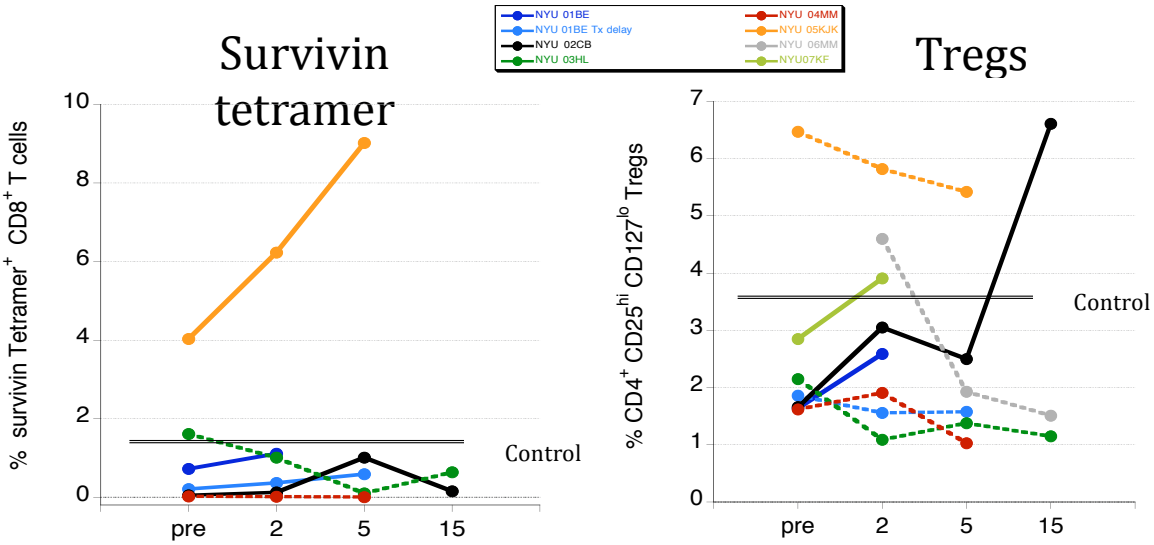


Fig 2: Tetramer and Treg trends in NYU patients 1-7.

These data are still preliminary and require further analysis, but 1 of the 5 patients that could be analyzed for tumor-specific responses demonstrated effector T cells prior to tratment that increased dramatically with time. This same patient also had the highest regulatory T cells (Tregs) and these decreased over the same time period. This can clearly be considered to be a treatment-induced immune response. One patient had a dramatic increase in Tregs at 15 weeks after therapy, but in general Treg were low and tended to fall after therapy.

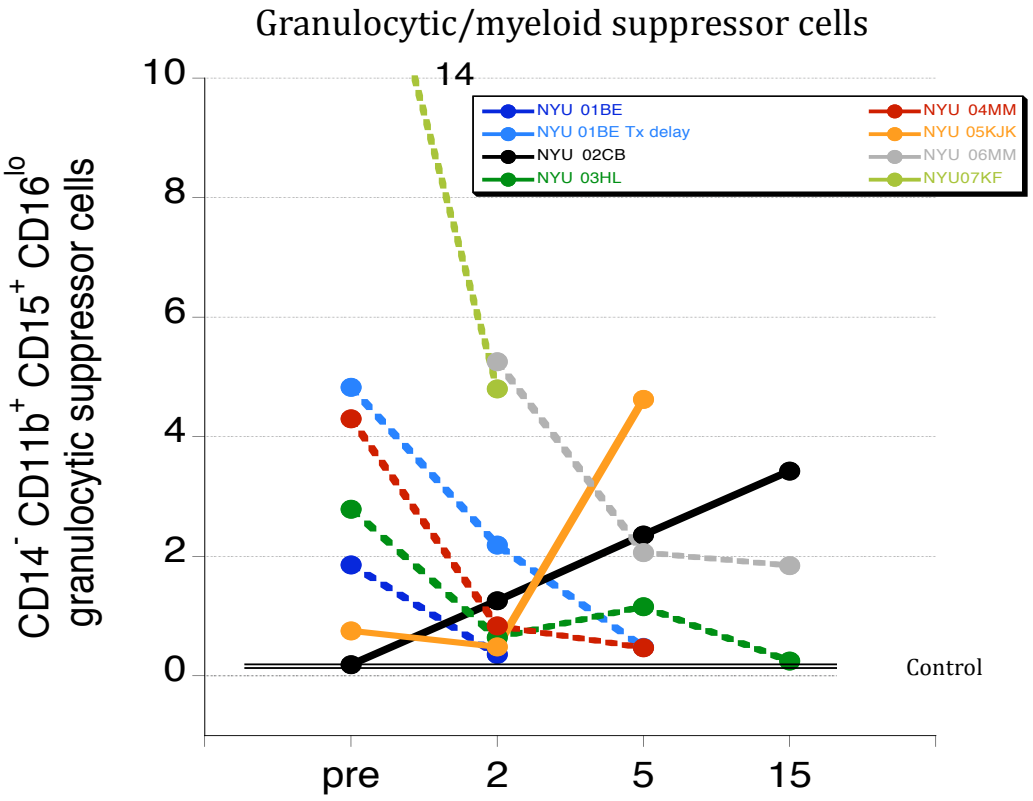


Figure 3: Granulocytic/myeloid suppressor cells in NYU patients 1-7.

The other immune cell subsets did not show any remarkable change and will not be reported here, with the exception of the granulocytic/myeloid suppressor cells (Fig 3). Practically all patients had high levels of granulocytic/myeloid suppressor cells prior to treatment and most fell dramatically during therapy. The 2 exceptions were the patient with high tumor-specific T cells and the other (#2) also had increasing levels of Tregs.

These analyses should not be considered as being final and complete as they have been performed only since the last report. However, they are very encouraging. We are clearly able to show clear differences in responses between patients receiving Fresolimumab+RT. We obviously do not know the dose of Fresolimumab that patients received and no attempt has as yet been made to correlate any of these responses with imaging responses or outcome. The fact that we do see these differences however gives us hope that we will be able to perform these correlations when the time comes.

2.2 Effects of DnTGF-beta receptor

An off-study publication (Quatromoni et al Translational Medicine 10:217, 2012) that we were recently involved in provides a good demonstration of the manner in which TGF-beta acts to inhibit anti-tumor immunity. Transgenic Pmel-1 CD8+ve T cells were rendered insensitive to TGF-beta signaling with a Dn TGF-beta dominant negative receptor II. These cells were more effective at mediating regression of an established B16 melanoma than non-transduced cells, providing proof of prinicple that TGF-beta is able to inhibit T cell anti-tumor responses, inhibiting active interferon-gamma producing T cells. (Fig 4)

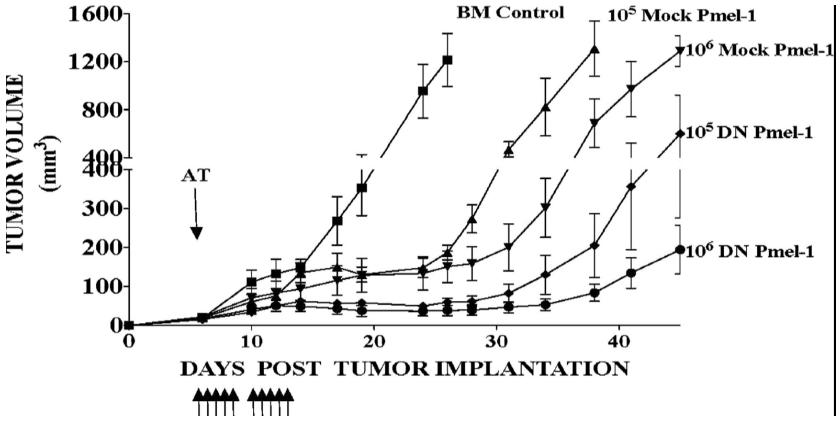


Figure 4: Dn TGF-beta T cells are able to mediate B16 tumor regression more effectively than control mock-tranduced T cells

3.0 Effects of TGF-beta on breast cancer stem-cells

Recent preclinical and clinical data support that solid cancers including breast cancers are organized hierarchically with a small population of cancer stem cells (CSCs), capable of re-growing the entire tumor while their progeny lack this ability. Furthermore, we and others reported that breast CSCs (BCSCs) are relatively resistant to ionizing radiation. To explain this enrichment, we first demonstrated that CSCs were resistant to radiation by bypassing radiation-induced apoptosis and/or senescence. After radiation, the surviving BCSCs were recruited from a quiescente state (G0) into an active cell cycle, allowing repopulation of the tumor. We also demonstrated that radiation treatment could induced reprogramming of non-tumorigenic cancer cells to generate new cancer stem cells (induced cancer stem cells, iCSCs). The mechanisms involved require the re-expression of the stemcell transcription factors Oct-4 Sox2 and Nanog. Interestingly, this re-expression was higher in polyploid cells.

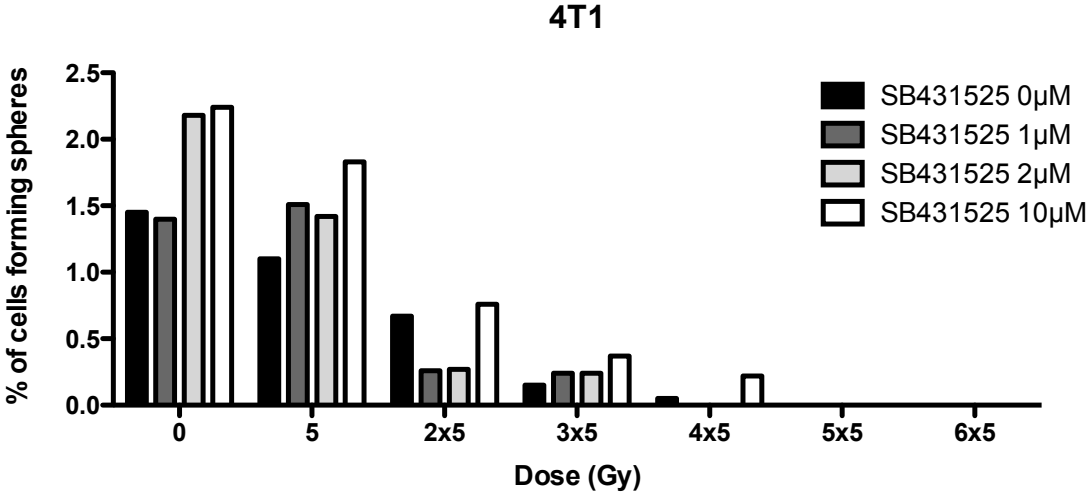
Furthermore, we identified TGFβ activation as a regulator of BCSCS expansion. Indeed, we also observed considerable enrichment for BCSCs when breast cancer cells were treated with an inhibitor of the TGFβ receptor. This enrichment could not be explained by a simple differential cell killing. Inhibition of TGFβ could induce expression of a key

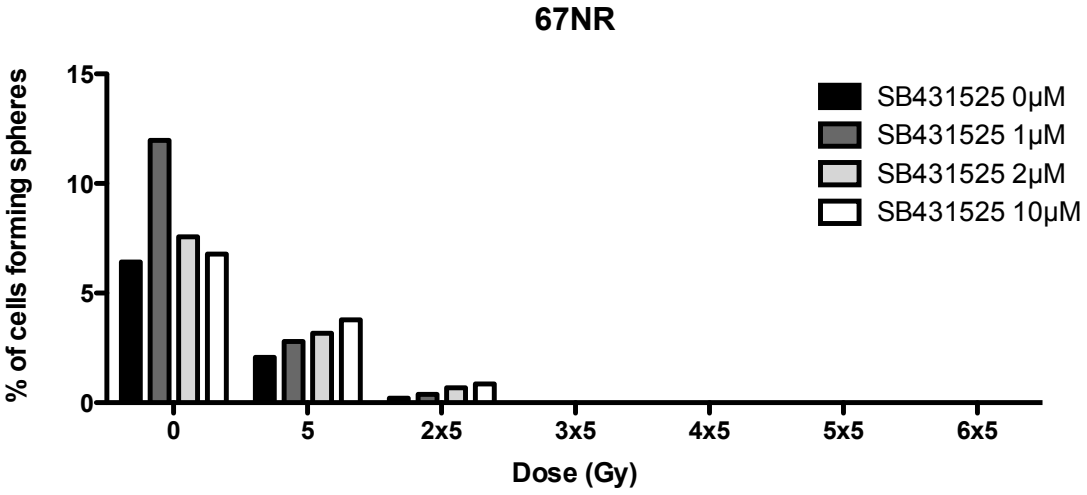
stem cell signaling pathway: Notch, which may explain why TGFβ inhibition could also potentiate reprogramming.

All previous experiments (enrichment and qRT-PCR analysis) were done with human breast cancer cell lines. Following the aims in the grant proposal, we switched to a mouse breast cancer cell line, which allows use of a syngeneic tumor model in vivo. 67NR and 4T1 cells lines were therefore explored as a model for the effects of TGFβ inhibition of the cancer stem cell population.

We plated 67NR and 4T1 cells in monolayer conditions. Cells were then pre-treated with 0, 1, 2 or 10 μM of TGFβ inhibitor (SB431525) before irradiation with 5 Gy, for 6 consecutive days. 72 h after the last dose, cells were trypsinized and plated for sphere forming capacity. The number of the spheres growing in each well was counting under microscope and relative percentage of cancer stem cells were enumerated (Figure 1).

Interestingly, the 2 cells lines show differential radiosensitivity. While 67NR exhibited less capability to form spheres at 0Gy (1.5%, 6% for 4T1), this cell line seems to be more resistant to fractionated radiation than 4T1. The effect of the TGFβ inhibitor, SB431525, on sphere formation so far seems to be less in mouse than in human breast cancer cell lines. Nevertheless, TGFβ inhibition enriched for cancer stem cells.





3. Problems Encountered

There have been no problems with subject enrollment or retention. We have received all subjects from the UCLA Department of Hematology-Oncology as referrals. There are no problems to report regarding the conduct of study procedures, consenting, confidentiality or anything else that would be considered reportable. All SAEs and AEs have been reported to NYU and the UCLA IRB as per protocol.

4. Future directions

We believe we are well on track to meet our goal of enrolling 14 subjects at UCLA within the next 6 months.

We will continue to receive frozen PBMCs from NYU and run these for immune monitoring but our immediate priority is to start the immune monitoring of the UCLA patients.

The anti-TGF-beta studies with cancer stem cells will continue to investigate the importance of this pathway in reprogramming.

5. Key Research Accomplishments

- Trial recruitment is proceeding well
- Immune monitoring has been standardized and the intial data indicate no unexpected problems.
- TGF-beta affects non-stem cell reprogramming by radiation exposure